Genomic prospecting for microbial biodiesel production

Athanasios Lykidis*
Genome Biology Program
DOE-Joint Genome Institute
2800 Mitchell Drive
Walnut Creek, CA 94598
Tel: 925-296-5842
Fax: 925-296-5840
Email: alykidis@lbl.gov

Natalia Ivanova
Genome Biology Program
DOE-Joint Genome Institute
2800 Mitchell Drive
Walnut Creek, CA 94598
Tel: 925-296-5832
Fax: 925-296-5840
Email: nnivanova@lbl.gov

*Corresponding author. Send proofs to:

Athanasios Lykidis
Genome Biology Program
DOE-Joint Genome Institute
2800 Mitchell Drive
Walnut Creek, CA 94598

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Abstract

Biodiesel is defined as fatty acid mono-alkylesters and is produced from triacylglycerols. In the current article we provide an overview of the structure, diversity and regulation of the metabolic pathways leading to intracellular fatty acid and triacylglycerol accumulation in three types of organisms (bacteria, algae and fungi) of potential biotechnological interest and discuss possible intervention points to increase the cellular lipid content. The key steps that regulate carbon allocation and distribution in lipids include the formation of malonyl-CoA, the synthesis of fatty acids and their attachment onto the glycerol backbone, and the formation of triacylglycerols. The lipid biosynthetic genes and pathways are largely known for select model organisms. Comparative genomics allows the examination of these pathways in organisms of biotechnological interest and reveals the evolution of divergent and yet uncharacterized regulatory mechanisms. Utilization of microbial systems for triacylglycerol and fatty acid production is in its infancy; however, genomic information and technologies combined with synthetic biology concepts provide the opportunity to further exploit microbes for the competitive production of biodiesel.

1. Introduction

Biodiesel is defined as the mono-alkyl esters of long chain fatty acids and constitutes an alternative fuel for compression-ignition (diesel) engines. The most common biodiesel constituent used today is fatty acid methyl etsters. Currently, the origin of fatty acids is triacylglycerols (TAGs) recovered from vegetable oils and animal fats. Biodiesel is non-toxic, completely biodegradable fuel with reduced sulfur emissions. It is produced in various countries on a large scale. The main source of biodiesel in the U.S is soybean oil and yellow grease whereas in Europe and Asia it is rapeseed and palm oil respectively. In all cases, the current and projected production cost of biodiesel is 2-3 times higher than the petroleum based product. Based on these projections it has been assumed that biodiesel can not be a competitive alternative to petroleum based fuel. However, microbial metabolic engineering presents a unique opportunity to lower the costs associated with the raw materials used in biodiesel production.

Biodiesel production from plant oils encounters certain limitations regarding mainly the availability of oil-seed supplies in suitable quantities and at competitive prices. On the contrary, the use of microbial systems for biodiesel production, although not exploited industrially until now, holds the promise to overcome these limitations. In addition, microbes can be tailored to utilize various carbon sources as feedstock for the production of oils, such as waste or agricultural byproducts. The current article will focus on the structure and regulation of the pathways utilized by various microbes (bacteria, algae, and yeast) for the production of fatty acids and TAGs (Figure 1).

From 1978 to 1996 the U.S. Department of Energy supported the "*Aquatic Species Program: Biodiesel from Algae*" [http://www1.eere.energy.gov/biomass/pdfs/biodiesel_from_algae.pdf] focused on the production of biodiesel from algae grown in ponds, utilizing waste CO_2 from coal fired power

plants. The program generated a collection of approximately 300 oil-producing species, mostly green algae and diatoms. It also explored the commercial utilization of these organisms in open pond production systems. Funding for this program ceased in 1995 due to budget limitations. More than a decade later we can see that the major limitation of the program has been the absence of genetic and molecular tools for exploration and manipulation of the genes, pathways and regulatory elements responsible for oil accumulation in these species. The program ended at the same period when major inroads in understanding the genes and regulatory mechanisms operating in lipid metabolism were happening in other organisms. Therefore, improvement of the performance of these algal cultures by manipulating the key enzymes and regulators and/or knocking out catabolic pathways could not be approached at the time of the *Aquatic Species Program*. At present, we already have whole genome sequences of seven algal species and more genomes are expected to be sequenced in the next few years. These data should provide the foundation for a better understanding of the biosynthetic pathways and regulatory networks of lipid metabolism in these organisms.

2. Lipid-accumulating microbes.

Biosynthesis and accumulation of TAGs has been reported in a number of microbes including bacteria, algae and yeast. The buildup of TAG in bacteria has been observed mainly in organisms belonging to the actinomycetes group, such as *Mycobacterium*, *Streptomyces*, *Rhodococcus* and *Nocardia* (Alvarez and Steinbuchel, 2002). *Streptomyces* species accumulate TAGs during the post exponential phase to concentrations ranging between 50-150 mg/l medium (Olukoshi and Packter, 1994). Other actinomycetes also accumulate TAGs and may contain up

to 8-20% of cellular dry weight in fatty acids (Alvarez and Steinbuchel, 2002). A separate group of gram-negative bacteria has been shown to accumulate TAG and wax esters (esters of long-chain fatty alcohols and fatty acids). For instance, cells of *Acinetobacter* species accumulate large amounts of wax esters when they grow in a nitrogen-limited medium in the presence of alkanes or alkanols (Ishige *et al.*, 2002).

In addition to bacteria, two classes of eukaryotic microbes contain organisms with remarkable abilities to accumulate lipids. Certain fungi, such as *Mortierella ramanniana*, species of *Lipomyces* and *Rhodotorula*, can accumulate substantial amounts of TAGs above 50% of their dry weight (Ratledge and Wynn, 2002), Three genomes of oleaginous fungi have been completely sequenced: *Aspergillus nidulans* (Galagan *et al.*, 2005), *Debaryomyces hansenii* (Dujon *et al.*, 2004), and *Yarrowia lipolytica* (Dujon *et al.*, 2004) and they will serve as our paradigms in this review. These organisms accumulate approximately 20-30% of their cellular mass as lipids.

Besides fungi, algae have been long known to accumulate lipids and particularly TAGs (Guschina and Harwood, 2006). Algae have attracted particular attention for biotechnological applications and several efforts are under way to exploit their utility in biodiesel production. The effect of various growth parameters (nutrient limitation, CO_2, light intensity) on the lipid composition of several algal species has been studied. Factors that favor increased intracellular TAG levels include nutrient limitation, increased CO_2 concentration and increased light density. Silicon, nitrogen or phophorus limitation caused an increase in TAG content and a concomitant decrease of polar lipids in the freshwater diatom *Stephanodiscus minutulus* (Lynn *et al.*, 2000). Growth of algae (*Tichocarpus crinitus*) at high light densities resulted in a 50% increase in the levels of intracellular TAGs (Khotimchenko and Yakovleva, 2005). The same correlation

between light intensity and TAG accumulation was observed in the green filamentous alga *Cladophora* spp. (Napolitano, 1994). TAGs were also accumulated in *Chlamydomonas* sp. grown in medium at pH 1 compared to cells grown at higher pHs (Tatsuzawa *et al.*, 1996). Data from seven algal genomes are currently available: the red algae *Cyanidioschyzon merolae* (Matsuzaki *et al.*, 2004) and *Galdieria sulphuraria* (Barbier *et al.*, 2005), the diatoms *Thalassiosira pseudonana* (Armbrust *et al.*, 2004) and *Phaeodactylum tricornutum* (unpublished; sequence data available at http://www.jgi.doe.gov), the chlorophytes *Ostreococcus tauri* and *Ostreococcus lucimarinus* (Derelle *et al.*, 2006; Palenik *et al.*, 2007), and *Chlamydomonas reinhardtii* (unpublished; sequence data available at http://www.jgi.doe.gov).

3. Fatty acid synthesis.

3.1 Initiation of fatty acid synthesis. Acetyl-CoA is a central metabolite synthesized from pyruvate. Acetyl-CoA carboxylase (ACC) is the committed step in fatty acid biosynthesis and constitutes the entrance gate of carbon towards cellular lipids. It catalyzes the ATP-dependent conversion of acetyl-CoA to malonyl-CoA. Acetyl-CoA is a key metabolite and a branching point in intracellular carbon distribution. Bacterial ACC is heteromeric (type-II ACC) and is composed of four subunits encoded by distinct genes: the biotin carboxyl carrier protein, *accB*; biotin carboxylase *accC*; and two proteins catalyzing the carboxyltransferase reaction *accA* and *accD* (Cronan and Waldrop, 2002).

The ACC subunits described above are fused into a single polypeptide encoded by one gene (type-I ACC) in higher eukaryotes resulting in a homomeric enzyme form. The *S. cerevisiae* genome encodes two ACC enzymes. The first (ACC1) is a 2233 amino acid long protein and it is located in the cytosol (Al-Feel *et al.*, 1992; Hasslacher *et al.*, 1993), whereas the

second (Hfa1) is targeted to mitochondria and is approximately 2120 amino acids long (Hoja *et al.*, 2004). The two yeast ACC isoforms appear to have unique functions in yeast physiology since mutations in the two genes have different phenotypes and the genes do not complement each other. *ACC1* is involved in cytoplasmic fatty acid biosynthesis and its disruption is lethal and cannot be compensated by external fatty acids. On the contrary *HFA1* mutants are viable, but fail to grow on lactate and glycerol and have reduced intracellular levels of lipoic acid (Tehlivets *et al.*, 2007). Analysis of the genomes of *A. nidulans, D. hansenii* and *Y. lipolytica* demonstrates that each of them contains only one ACC isoform more closely related to the cytosolic yeast ACC1 (locus tags AN6126.2, DEHA0B05632g and YALI0C11407g, respectively).

In contrast to yeasts, many plants contain both ACC forms, the heteromeric form in the plastid and the homomeric form in the cytosol (Sasaki and Nagano, 2004). The only isolated algal ACC gene, that of *Cyclotella cryptica* (Roessler and Ohlrogge, 1993), and codes for a single polypeptide of 2089 amino acids. However, ACC homologs are easily identifiable in all available algal genomes: *T. pseudonana, P. tricornutum* and *O. tauri* genomes encode two large ACC polypeptides whereas *C. merolae* and *G. sulphuraria* both have only one type-I ACC homolog in the available data. Notably, *C. rheinhardtii* is missing type-I ACC homologs, but it has a type-II heteromeric ACC. The ACC1 isoform of *T. pseudonana* contains a chloroplast targeting sequence at the amino terminus of the predicted coding sequence model whereas the ACC2 predicted protein sequence is not complete at the respective amino terminal region.

Several lines of evidence from different organisms suggest that the ACC step is a key regulator of carbon flow towards fatty acids and lipids. In bacteria, overexpression of ACC activity increases the flow of carbon though the fatty acid-lipid biosynthesis branch of cellular

metabolism (Davis *et al.*, 2000). Overexpression of *Arabidopsis* ACC in the amyloplasts of potato tubers increased fatty acid synthesis and led to a 5-fold increase in the amount of accumulated TAGs (Klaus *et al.*, 2004). On the contrary, down-regulation of the ACC1 expression in *Brassica napus* resulted in lower lipid content (Slabas *et al.*, 2002). Although the effect was not substantial (lipid levels in mutant strains were only 10-30% lower than in the wild type plants), the results were consistent with the proposed role of this enzymatic step in regulating carbon flux towards fatty acids.

The regulation of bacterial ACC has been studied in *E. coli* and *B. subtilis*. Transcription levels of the four *acc* genes in *E. coli* and *B. subtilis* are directly correlated with the growth rate (Li and Cronan, 1993; Marini *et al.*, 2001). However, *accBC* expression from the native promoter did not depend on gene copy number suggesting a transcriptional regulation mechanism. Subsequent work demonstrated that the AccB protein functions as an autoregulator of the *accBC* operon in *E. coli* suppressing its expression (James and Cronan, 2004).

In yeast ACC activity is regulated at multiple levels. Acc1 gene expression is controlled in coordination with membrane phospholipid biosynthesis by the Ino2/Ino4 and Opi1 transcription factors (Hasslacher *et al.*, 1993). In addition, posttranslational modification by phosphorylation appears to inactivate yeast ACC *in vivo* (Witters and Watts, 1990). The *S. cerevisiae* Ino2/Ino4 and Opi1 transcription factors are major regulators of lipid metabolism and control the expression of multiple genes such as fatty acid synthases and phospholipid biosynthesis genes (Carman and Henry, 1999). They exert their function by binding to UAS_{INO} (Upstream Activating Sequences INO) elements. Ino2 and Ino4 form a heterodimeric activator complex that binds the 10-bp UAS_{INO} element. Opi1 is a negative regulator required for the repression of UAS_{INO} associated genes. Opi1 is retained in the ER by forming a complex with

the membrane protein Scs2p. Intracellular levels of phosphatidic acid (PtdOH) act as a signal regulating the translocation of Opi1 to the nucleus. Opi1 binds PtdOH and high concentrations of PtdOH in the ER membrane are required for its retention in the ER. However, when PtdOH concentrations drop Opi1 is released and translocated to the nucleus where it represses the transcription of lipid biosynthetic genes. *A. nidulans*, *D. hansenii* and *Y. lipolytica* contain proteins (AN8817.2, DEHA0G03894g and YALI0C14784g, respectively) with approximately 25% identity to *S. cerevisiae* Opi1. The relatively low similarity score does not allow confident prediction that these proteins are involved in the regulation of lipid metabolism in a manner similar to Opi1. Furthermore, there are no proteins with significant similarity to Ino2p and Ino4p in *A. nidulans*, *D. hansenii* and *Y. lipolytica* suggesting the existence of a divergent set of regulators controlling fatty acid and lipid metabolism in these oleaginous fungi.

3.2 Elongation steps of fatty acid synthesis. Two distinct enzyme systems catalyzing the synthesis of fatty acids have been described. The type-I systems (FASI), typically found in yeasts and mammals, include large multifunctional proteins harboring all the necessary enzymatic activities in one polypeptide. On the contrary, the type-II systems are composed of monofunctional proteins. Fatty acid biosynthesis in most bacteria proceeds with the type-II, or dissociated, system (FASII). In both systems fatty acid biosynthesis involves five distinct enzymatic steps: 1) the first step, catalyzed by malonyl-CoA:ACP transacylase, transfers the malonyl group from CoA to acyl carrier protein (ACP) to form malonyl-ACP which serves as an immediate donor of the two-carbon acetyl units used in fatty acid elongation; 2) the condensing step, catalyzed by β-ketoacyl-ACP synthases, adds the two-carbon unit to the growing acyl-ACP; 3) the NADPH-dependent reduction step catalyzed by β-ketoacyl-ACP reductases yields β-

hydroxyacyl-ACP; 4) the dehydration step catalyzed by β-hydroxyacyl-ACP dehydrases yielding *trans*-2-enoyl-ACP; and 5) the last reductase step catalyzed by enoyl-ACP reductases which forms a saturated acyl-ACP serving, in turn, as the substrate for another condensation reaction (Marrakchi *et al.*, 2002). A repetitive series of reactions follows adding two-carbon units per cycle, until a saturated fatty acid of 16-18 carbons is formed. Some *Actinobacteria* e.g. *Mycobacteria*, *Corynobacteria*, *Nocardia* utilize both FASI and FASII systems. Work in *Mycobacteria* has shown that the FASI system is utilized for the production of fatty acids up to 18 carbon long and FASII elongates them to 26-30 (Schweizer and Hofmann, 2004). However, several *Actinobacteria* genera of industrial interest e.g. *Streptomyces* do not have a FASI system and utilize exclusively the dissociated pathway. Several recent reviews summarize the current understanding of the genes that participate in bacterial fatty acid synthesis (Campbell and Cronan, 2001b; Zhang *et al.*, 2003).

The type-II system is also present in plants and it is localized in the plastid although all of its components are encoded by the nuclear genome (Ohlrogge and Jaworski, 1997). Similar to plants all available algal genomes (*C. merolae*, *T. pseudonana*, *P. tricornutum, O. tauri*, *O. lucimarinus* and *C. rheinhardtii)* encode homologs of the type-II components indicating the presence of the dissociated system for fatty acid biosynthesis in these organisms and its probable localization to the chloroplast.

While most higher eukaryotes utilize a type-I system (FASI) containing all five activities in a single large polypeptide, fungi have these activities located on two polypeptides, as exemplified by the FAS1 (β subunit) and FAS2 (α subunit) enzymes of *S. cerevisiae* (Tehlivets *et al.*, 2007). The yeast FAS holoenzyme is a hexameric complex α6β6located in the cytosol. Homologs of the *S. cerevisiae* FAS1 and FAS2 subunits are easily recognized in the genomes of

D. hansenii, *Y. lipolytica* and *A. nidulans*. In addition, *S. cerevisiae* contains a complete type-II fatty acid synthesis system localized in mitochondria; products of this fatty acid synthase are utilized for lipoic acid formation and they do not appear to be significant contributors to phospholipid and TAG formation.

3.3 Termination steps of fatty acid biosynthesis.

Fatty acid biosynthesis systems have evolved to produce acyl chains approximately 16-18 carbon atom long; longer fatty acids are produced in many organisms by specific systems (fatty acid elongases). In bacteria there is no specific mechanism for terminating acyl chain elongation; when acyl-ACP reaches 16 or 18 carbons it becomes a substrate for the acyltransferases that will attach the fatty acyl chain onto the glycerol backbone to produce phospholipids. However, plants have a special class of enzymes that will terminate the acyl chain elongation by hydrolyzing the thioester bond of acyl-ACP thus releasing free fatty acids and ACP. This enzyme is known as *f*atty acyl-*A*CP *t*hioesterase (FAT) and is localized in the plastid. Free fatty acids produced by FAT exit the plastid and are re-esterified with CoA. The resulting acyl-CoAs are utilized for glycerolipid biosynthesis in the ER. Based on sequence similarity and substrate specificity two classes of thioesterases have been described: the FATA class which is active on unsaturated acyl-ACPs and the FATB class which prefers saturated acyl groups (Salas and Ohlrogge, 2002). Unlike higher plants that contain multiple FAT genes, the genomes of algae *C. reinhardtii*, *O. tauri* and *O. lucimarinus* contain only one FAT homolog, while *C. merolae* and the diatoms *T. pseudonana* and *P. tricornutum* have no FAT homologs in the current assemblies, so the mechanism of acyl chain termination in these algae is unknown.

An important observation with regard to the possibility of microbial biodiesel production has been made when FAT enzymes were overproduced in *E. coli*. Expression of a plant (*Umbellularia californica*) medium-chain FAT in an *E. coli* strain deficient in fatty acid oxidation resulted in a four-fold increase of the fatty acid output of the bacterial culture (Voelker and Davies, 1994). Thus far it represents the most efficient way to uncouple fatty acid formation from phospholipid and membrane biosynthesis in *E. coli*. The resulting fatty acids were enriched in acyl chains of 14 carbon atoms indicating that the production of fatty acids with specific acyl length may be feasible using enzymes with the appropriate specificity. Lowering the acyl chain length of the fatty acid esters may have substantial effects on the physical properties and overall performance of the resulting fuels.

3.4 Regulation of fatty acid biosynthesis.

Fatty acid synthesis is regulated at multiple points and different mechanisms operate to provide transcriptional and biochemical control of the carbon flow towards lipids. The regulation of fatty acid biosynthesis has been studied in *E. coli* and *B. subtilis* which serve as models for gram negative and gram positive bacteria, respectively. Extensive work in *E. coli* demonstrated that fatty acid biosynthesis is regulated coordinately with phospholipid biosynthesis and growth in response to changes in the environment. Fatty acid biosynthesis ceases when bacteria enter the stationary phase. This response is mediated by the intracellular levels of ppGpp (guanosine 5-diphosphate-3-diphophate). Elevated levels of ppGpp *in vivo* inhibit PlsB, the first acyltransferase catalyzing the attachment of acyl groups to glycerol-3-phosphate (Heath *et al.*, 1994). The inhibition of this first step of PtdOH formation leads to elevated levels of acyl-ACPs (Heath and Rock, 1996a; Rock and Jackowski, 1982) which, in

turn, serve as feedback inhibitors of three key enzymes in the pathway. The ACC reaction is inhibited by long-chain acyl-ACPs (Davis and Cronan, 2001) resulting in a decreased flux of malonate groups into the pathway. The first enzyme in fatty acid biosynthesis, FabH, is also inhibited by acyl-ACPs (Heath and Rock, 1996a, 1996b) resulting in the attenuation of fatty acid biosynthesis initiation. Finally, acyl-ACPs inhibit the last enzyme in fatty acid elongation, FabI, slowing the overall rate of fatty acid formation (Heath and Rock, 1996a). Fatty acid biosynthesis can be uncoupled from phospholipid synthesis and growth stage by overexpression of thioesterases (Cho and Cronan, 1995; Dormann *et al.*, 1995; Jiang and Cronan, 1994; Ohlrogge *et al.*, 1995; Voelker and Davies, 1994). Thioestereases hydrolyze the acyl-ACP thioester releasing free fatty acid and ACP terminating the acyl elongation cycle. The resulting free fatty acids are secreted en masse to the medium and these strains are very good candidates for further exploration with regard to biodiesel production.

Transcriptional regulation of bacterial fatty acid biosynthesis is, in general, poorly understood. There are two well-documented examples of transcriptional regulation of bacterial fatty acid biosynthesis, one in gram-negative and one in gram-positive bacteria. In *E. coli*, two TetR-type transcriptional regulators, the activator FadR and the repressor FabR, have been shown to regulate the expression of *fabA* and *fabB* genes. The FadR was initially discovered as a repressor of fatty acid degradation genes (DiRusso and Nunn, 1985; Overath *et al.*, 1969) and was subsequently shown to be an activator of *fabA* (Henry and Cronan, 1992) and *fabB* (Campbell and Cronan, 2001a). Long-chain acyl-CoAs regulate the DNA binding activity of FadR (DiRusso *et al.*, 1992; DiRusso *et al.*, 1998; Raman and DiRusso, 1995). The second *E. coli* regulator, FabR, acts as a repressor of the *fabA* and *fabB* genes (Zhang *et al.*, 2002) but it is currently unclear which ligand(s) modulates its activity. FadR and FabR have similar

phylogenetic distribution and their homologs can be found in the species of *Escherichia*, *Salmonella*, *Yersinia*, *Vibrio*, *Haemophilus*, and *Shewanella*.

A separate transcriptional system regulating the expression of FASII enzymes has been described in *B. subtilis* and is mediated by FapR (Schujman *et al.*, 2003). Subsequent work demonstrated that malonyl-CoA functions as the direct and specific signal for the control of FapR activity (Schujman *et al.*, 2006). Binding of malonyl-CoA, the key intermediate in fatty acid formation, promotes dissociation of the FapR-DNA complex resulting in transcription of FASII genes. FapR homologs can be identified in various gram-positive bacteria, such as *Bacilli*, *Listerias* and *Staphylococci*. There is no substantial information on the regulation of fatty acid biosynthesis in most bacteria including cyanobacteria, actinobacteria etc.

Most of the information regarding the regulation of FAS1 and FAS2 gene expression in fungi comes from the studies in *S. cerevisiae*. In this organism FAS1 and FAS2 expression is controlled by the lipid precursors choline and inositol through UAS_{INO} sequences (Schuller *et al.*, 1992). In addition, *FAS1* and *FAS2* genes are activated by the general transcription factors Gcr1, Abf1p Rap1p and Reb1 and contain the respective binding sites in their promoter regions (Greenberg and Lopes, 1996; Schuller *et al.*, 1994). Analysis of *A. nidulans*, *D. hansenii* and *Y. lipolytica* genomes indicate that these oleaginous fungi do not harbor proteins with significant similarity to the *Saccharomyces* Gcr1p and Abf1p transcription factors. However, they contain homologs of the Reb1 regulator (AN4618.2, DEHA0E13266g and YALI0F20460g, respectively) and *D. hansenii* contains a protein (DEHA0C13959g) with relatively low similarity score (expectation value of -30) to Rap1p. There is no experimental information on the regulators of fatty acid synthesis in these oleaginous fungi and the above genomic observations are consistent with the hypothesis that despite the high similarity in the structure and organization of the FAS

genes their regulation may vary significantly among organisms. An interesting observation regarding the interrelationship between the *Saccharomyces FAS1* and *FAS2* genes refers to the dependence of *FAS2* mRNA abundance on *FAS1* expression (Wenz *et al.*, 2001). Overexpression of FAS1p increases *FAS2* levels whereas *FAS1* mutants have decreased levels of *FAS2* mRNA. On the contrary, *FAS1* expression is independent of *FAS2* expression. The responsible regulatory site is located within the coding sequence of *FAS2*. Whether this auto-regulatory mechanism is conserved in other fungi remains to be established.

3.5 Unsaturated fatty acid biosynthesis

Besides acyl chain length, the saturation degree of the fatty acids plays an important role in the physical properties of the resulting biodiesel. Particularly, it influences the viscosity in low temperatures. *cis*-Unsaturated and polyunsaturated fatty acids (UFAs and PUFAs) play vital roles in membrane biology and their relative content influences the physical properties of most membrane systems regulating the fluidity of cellular membranes. Two major mechanisms have been described that synthesize UFAs: the oxygen dependent fatty acid desaturation pathway which has representatives in both bacteria and eukaryotes and the anaerobic pathway which inserts the double bond concomitantly with the elongation of the acyl length as exemplified by the *E. coli* system. The oxygen-dependent dehydrogenation at a specific position of the acyl chain is catalyzed by fatty acid desaturases and results in a *cis*-double bond. Fatty acid desaturases with specificity for different positions of the acyl chain have been described in a variety of organisms; for instance, *B. subtilis* has a Δ5 (acting on the fifth carbon of the acyl chain) desaturase (Aguilar *et al.*, 1998). The biotechnological potential of fatty acid desaturases for the synthesis of UFAs and PUFAs has been studied extensively (Huang *et al.*, 2004; Qi *et al.*,

2004). Fatty acid desaturases are particularly abundant in soil and aquatic bacteria. All cyanobacteria that have been sequenced to date have multiple desaturases in their genomes which have presumably distinct specificities and potentially resulting in synthesis of polyunsaturated fatty acids. A two-component regulatory system that controls expression of the *B. subtilis* desaturase at low temperatures has been described (Aguilar *et al.*, 2001). The histidine kinase DesK senses changes in membrane fluidity caused by low temperatures or other factors and activates the response regulator DesR which induces transcription of the desaturase (Aguilar and de Mendoza, 2006). Close homologs of the DesK-DesR system can be identified in other *Bacilli* but not in other organisms suggesting the existence of divergent (at least in terms of sequence similarity) regulatory systems.

S. cerevisiae has a single fatty acid desaturase, Ole1p, generating a double bond in the Δ9 position of fatty acids (Stukey *et al.*, 1989). *A. nidulans* and *D. hansenii* each have two fatty acid desaturase genes in their genomes (AN6731.2, AN4135.2 and DEHAOF04268g, DEHAOF25432g, respectively) whereas *Y. lipolytica* has one (YALI0C05951g). Transcription of *Ole1* in *S. cerevisiae* depends on two transcription factors, SPT23 and MGA2. Disruption of either gene has little effect on Ole1 whereas disruption of both genes results in auxotrophy for UFA (Zhang *et al.*, 1999). *A. nidulans*, *D. hansenii* and *Y. lipolytica* genomes encode proteins with relatively good similarity to the SPT23 and MGA2 proteins (20-30% amino acid identity); these homologs may be involved in regulation of UFA synthesis.

Multiple desaturases have been also characterized from plants and much work has been focused on their action and regulation because of the significant dietary value of polyunsaturated fatty acids. Multiple desaturases are easily recognized in the genomes of all algae sequenced to

date: *C. rheinhardti* has seven potential fatty acid desaturases, *T. pseudonana* thirteen, *O. tauri* eight, and *C. merolae* four.

4. Transfer of acyl groups to the membrane; Synthesis of PtdOH.

After they are formed, acyl groups are sequentially anchored onto the glycerol-3-phosphate backbone to form PtdOH. Two enzyme systems have been described that mediate the attachment of the first acyl group to generate 1-acylglycerol-3-phosphate (LysoPtdOH): the universal glycerol-3-phosphate acyltransferase (GPAT) system and the PlsX-PlsY acyl-phosphate-mediated system which appears to be confined in bacteria. GPAT catalyzes the initial acyl transfer from acyl-ACP onto glycerol-3-phosphate yielding LysoPtdOH. Recent work has uncovered an alternative route for the synthesis of LysoPtdOH in bacteria via the PlsX-PlsY pathway (Lu *et al.*, 2006). PlsX catalyzes the reaction between acyl-ACP and phosphate resulting in the formation of a novel intermediate, acyl-phosphate. PlsY subsequently transfers the acyl group from acyl-phosphate to glycerol-3-phosphate yielding LysoPtdOH. The PlsX-PlsY system for lipid biosynthesis is abundant in bacteria whereas the GPAT reaction appears to be limited to a few gram-negative genera and *Actinobacteria*. The absence of PlsX-PlsY homologs in *Actinobacteria* suggests that many oleaginous bacteria utilize exclusively the GPAT reaction for the initiation of TAG buildup.

The GPAT reaction is the only route described in eukaryotes for the association of the acyl groups with the glycerol backbone. LysoPtdOH is subsequently acylated to yield PtdOH in a step catalyzed by 1-acyl-glycerol-3-phosphate acyltransferase (AGPAT). *S. cerevisiae* contains two genes encoding GPAT activities, YKR067w and YBL011w. *D. hansenii* has also two homologous genes whereas *Y. lipolytica* and *A. nidulans* have only one GPAT gene. In *S.*

cerevisiae the second acyltransferase (AGPAT) is encoded by the *SLC1* gene and single copies of this gene can be found in the other fungal genomes.

Two classes of GPATs have been described in *A. thaliana*: one isoform is located in the plastid (Nishida *et al.*, 1993) whereas a separate gene family encompassing six members encodes extraplastidial GPATs (Zheng *et al.*, 2003) presumably residing on the endoplasmic reticulum. Five *Arabidopsis* genes code for AGPAT enzymes, one of which is targeted to the plastid (Kim and Huang, 2004). The compartmentalization of PtdOH synthesis in two separate organelles (plastid and ER) is a well-developed theme in plant lipid biology. Examination of the algal genomes indicates that they contain only the plastid versions of the two acyltransferases involved in PtdOH formation. *T. pseudonana, P. tricornutum, C. merolae, C. rheinhardtii,* and *O. tauri* are all missing recognizable extraplastidial GPAT homologs suggesting that either PtdOH synthesis occurs exclusively in the plastid or that the enzymes have dual localization both in the plastid and the ER. If the former hypothesis is true then lipid trafficking between the plastid and the ER would be important since the ER is the organelle where phospholipid and TAG synthesis occurs. Overexpression of plant and bacterial GPAT activities in *A. thaliana* resulted in increased seed oil content (Jain *et al.*, 2000). Also, overexpression of the yeast AGPAT in plants leads to 50% increase in TAG levels (Zou *et al.*, 1997). The above observations are consistent with the general theme that overexpression of any enzymatic step in the pathways leading to TAG increases the flow of carbon through the lipid pathway and causes accumulation of TAGs.

5. Triacylglycerol biosynthesis.

5.1 Phosphatidate phosphatases. PtdOH is a key branching point in *de novo* lipid metabolism and it is converted either to CDP-DAG or DAG depending on the organism. CDP-DAG and

diacylglycerol (DAG) serve as intermediates in membrane phospholipid biosynthesis and, in addition, DAG is converted to TAG. Phosphatidate phosphatases (PAPs) in coordination with phospholipid producing enzymes are key regulators of the flux of carbon towards TAGs. PAPs catalyze the conversion of PtdOH to DAG; the primary destination of DAG is the synthesis of membrane phospholipids whereas excess DAG is directed towards TAG. Recent work in *S. cerevisiae* has identified the PAP1 enzyme involved in *de novo* lipid biosynthesis as the yeast homolog of lipin (Han *et al.*, 2006). Lipin was first identified in mammalian cells as a regulator of lipid metabolism exerting a major effect on fat accumulation (Peterfy *et al.*, 2001; Phan and Reue, 2005). Lipin homologs can be identified in all eukaryotic genomes sequenced to date including algae and plants. *C. merolae, T. pseudonana* and *C. rheinhardtii* have one copy of the lipin gene in their genomes whereas *A. thaliana* has two copies.

The existence of PAP enzymes in certain bacteria is inferred by the structure of the biochemical pathways (Dowhan, 1997). For example, cyanobacteria synthesize DAG as an obligate intermediate in the formation of glycolipids and sulfolipids which are important molecules for the organization of the thylakoid membranes. Certain gram-positive bacteria also use DAG in the synthesis of glycolipids. However, a bacterial PAP has not been described and further research is needed to identify the genes coding for this activity.

5.2 Acyltransferases. Two types of reactions have been described that lead to formation of TAG: the first is acyl-CoA dependent, utilizes DAG and acyl-CoA and is catalyzed by diacylglycerol acyltransferase (DGAT) (Buhman *et al.*, 2001), whereas the second is acyl-CoA independent and uses phospholipid molecules as donors of the acyl group. The latter reaction is catalyzed by phospholipid: diacylglycerol acyltransferase (PDAT) (Dahlqvist *et al.*, 2000; Stahl *et al.*, 2004). The presence of DGAT and PDAT homologs in genomic sequences can serve as a

useful marker for the ability of various organisms to synthesize TAGs. DGATs have been characterized from various organisms including mammals (Buhman *et al.*, 2001), plants (Zou *et al.*, 1999), yeast (Sorger and Daum, 2002) and bacteria (Daniel *et al.*, 2004; Kalscheuer and Steinbuchel, 2003). In contrast, PDAT enzymes have been characterized in plants and yeast only (Dahlqvist *et al.*, 2000; Stahl *et al.*, 2004). In yeast the PDAT reaction is catalyzed by *LRO1* (*L*CAT *R*elated *O*pen reading frame) gene product. The relative contribution of the fungal DGAT and PDAT pathways to TAG accumulation was studied in *S. cerevisiae* utilizing *DGA1* and *LRO1* mutants. Overall, the results suggest that Lro1 contributes to TAG formation during exponential growth whereas Dga1 has significant participation in the stationary phase and during sporulation (Oelkers *et al.*, 2002). DGAT and PDAT homologs can be identified in *D. hansenii* and *Y. lipolytica* genomes suggesting the presence of both mechanisms for TAG formation in these organisms. On the contrary, characterization of an *A. thaliana* knockout line indicated that the PDAT enzyme is not a major contributor to seed TAG content (Mhaske *et al.*, 2005). DGAT enzyme levels are important regulators of the cellular TAG content: overexpression of *Arabidopsis* DGAT1 in tobacco leaves and yeast increases TAG levels (Bouvier-Nave *et al.*, 2000). Overexpression in *Arabidopsis* seeds also results in TAG accumulation (Jako *et al.*, 2001).

DGAT and PDAT homologs can be easily identified in all algal genomes sequenced to date, although they exhibit distinct distribution patterns. Examination of the algal genomic sequences reveals that *C. rheinhardtii* has three genes encoding for DGATs but it is missing a PDAT homolog. The diatoms *T. pseudonana* and *P. tricornutum* have both DGAT and PDAT homologs. On the other hand, *O. tauri* lacks recognizable DGAT homologs but it does have a

PDAT enzyme suggesting that TAGs are derived from phospholipids in this organism. C. merolae has DGAT but not a PDAT.

The first bacterial DGAT enzyme was identified and cloned in *Acinetobacter baylyi* sp ADP1 (Kalscheuer and Steinbuchel, 2003). In addition, it was shown that this enzyme is in fact bifunctional and it also catalyzes the reaction between acyl-CoA and fatty alcohol leading to the formation of wax esters (wax ester synthase, WS). DGAT/WS homologs can be identified in multiple bacteria from diverse phylogenetic origins. In addition to many strains of *Mycobacteria* and other soil *Actinobacteria* (*Nocardioides*, *Rhodococcus*, *Streptomyces*), DGAT homologs can be recognized in soil α-proteobacteria (*Bradyrhizobium japonicum*), aquatic and sediment β-proteobacteria (*Polaromonas* sp. JS666, *Rhodoferax ferrireducens* DSM 15236), aquatic γ-proteobacteria (*Photobacterium profundum*, *Psychrobacter arcticus*, *Psychrobacter cryohalensis* K5, *Hahella chejuensis* KCTC 2396, *Alkanivorax borkumansis*), aquatic bacteroidetes (*Salinibacter rubber* DMS 13855) and acidobacteria (*Solibacter usitatus* Ellin6076). Biochemical studies indicated that the *Acinetobacter* DGAT/WS can utilize acyl-CoAs of variable length and its utility in producing fatty acid esters was exploited by overexpression in different host backgrounds (Kalscheuer *et al.*, 2004; Stoveken *et al.*, 2005; Uthoff *et al.*, 2005). Coexpression of the *Acinetobacter* WS/DGAT with plant acyl-CoA reductase (which catalyzes the conversion of acyl-CoAs to fatty alcohols) in *E. coli* caused the production of wax esters up to 1% of the cellular dry weight (Kalscheuer *et al.*, 2006b). Expression of the above WS/DGAT in a *S. cerevisiae* strain lacking all four acyltransferases responsible for neutral lipid biosynthesis (TAGs and sterol esters) restored TAG formation but not sterol ester synthesis (Kalscheuer *et al.*, 2004). It also resulted in the formation of fatty acid ethyl esters and fatty acid isoamyl esters demonstrating the ability of this enzyme to utilize short-chain alcohols as substrates. This

remarkable property is directly applicable to biodiesel production. In a recent study, Kalscheuer et al (Kalscheuer *et al.,* 2006a) exploited *E. coli* as a host for the production of biodiesel. They coupled the expression of the *Acinetobacter* WS/DGAT with the expression of pyruvate decarboxylase and alcohol dehydrogenase from the anaerobic ethanologenic bacterium *Zymomonas mobilis*. Coexpression of the three genes resulted in intracellular accumulation of fatty acid ethyl esters (FAEEs) consisting mainly of ethyl oleate in concentrations up to 26% of bacterial dry biomass. However, the formation of FAEEs was strictly dependent on the presence of sodium oleate in the medium underlining the inability of the WS/DGAT system to utilize *de novo* formed fatty acids in *E. coli*. This result is another manifestation of the tight control of fatty acid biosynthesis and their destination in *E. coli*, which has to be uncoupled in order to channel fatty acids towards production of biodiesel. However, it also opens the door to the exploration of other bacterial species, particularly those able to amass fatty acids, as potential hosts.

The *Arabidopsis* AP2/EREBP transcription factor WRINKLED1 (WRI1) has been identified as a regulator of seed TAG accumulation (Focks and Benning, 1998) and it was named after the wrinkled appearance of the seed coat. Subsequent analysis indicated that *wri1* mutants are defective in a regulatory factor that controls the glycolytic breakdown of sugars and the availability of precursors for fatty acid biosynthesis and ultimately TAG accumulation (Cernac and Benning, 2004; Ruuska *et al.*, 2002) highlighting the interrelationship between carbon flow through glycolysis and lipid metabolism. WRI1 homologs can be identified in the genomes of algae *O. tauri, O. lucimarinus* and *C. rheinhardtii* but not in *G. sulfuraria, C. merolae, T. pseudonana* and *P. tricornutum*.

6. Correlation of lipid accumulation with the cellular redox status.

In addition to acetyl-CoA, fatty acid biosynthesis needs substantial amounts of NADPH to proceed. For the synthesis of every mol of C18 fatty acid there is a need for 18 mol of NADPH. Biochemical studies in oleaginous fungi suggested that malic enzyme (ME) is a critical step in supplying the necessary amount of NADPH for fatty acid synthesis occurring under conditions of nitrogen starvation, which induces lipid accumulation (Ratledge and Wynn, 2002; Ratledge, 2004). It has been suggested that instead of the traditional pyruvate-dependent mechanism, a distinct route for converting glucose to acetyl-CoA operates in oleaginous fungi. Under nitrogen limiting conditions cells activate AMP deaminase in an effort to scavenge additional nitrogen from intracellular resources. AMP deaminase converts AMP to inosine-5-monophosphate and ammonia. The decrease in AMP concentration has an inhibitory effect on isocitrate dehydrogenase, a TCA cycle enzyme, which requires AMP for its activity. The accumulating isocitrate is readily converted to citrate via aconitase and citrate is subsequently effluxed from the mitochondrion to the cytoplasm. Cytosolic citrate is converted to acetyl-CoA and oxaloacetate via the action of ATP-citrate lyase (ACL) which catalyzes the reaction

Citrate + CoA + ATP -> Acetyl-CoA + Oxaloacetate + ADP + Pi

The resulting acetyl-CoA is used for fatty acid biosynthesis whereas the oxaloacetate is converted to malate (via malate dehydrogenase) which enters the mitochondria as the counterion in the citrate efflux. The above route provides additional acetyl-CoA units for lipid buildup; in addition, malic enzyme supplies NADPH by catalyzing the reaction

Malate + $NADP^+$ -> Pyruvate + CO_2 + NADPH

The above schema is supported by observations in *A. nidulans* mutants lacking malic enzyme activity (Wynn *et al.*, 1999). The mutant cells accumulated only half the amount of lipids that

wild-type cells did. Further research is necessary to acquire a complete picture of the interrelationships and dependence of lipid biosynthesis on the overall redox state of the cell, especially in bacteria and algae.

7. Conclusions and perspective.

Our understanding of the biochemical and genetic steps that comprise and regulate lipid metabolism and oil accumulation has increased significantly during the last few years. The genes and pathways responsible for fatty acid and TAG biosynthesis have been identified in multiple model organisms. However, most of the work was performed with a mindset of either identifying potential drug targets in animal and plant pathogens or improving the nutritional value of agricultural products. A new end point of lipid research has to be introduced and this is the massive generation of fatty acids and/or TAGs that will serve as the raw material for biodiesel production.

Although the basic biochemical reactions utilized in lipid biosynthesis are essentially identical among the major domains of life, the multiple versions of enzymes, pathways and regulatory networks that participate indicate the fine tuning of these pathways to the environment. The emerging theme from genome comparisons underlines the evolution of distinct regulatory mechanisms in various phylogenetic groups. Carbon flow towards fatty acids occurs in order for the cell to synthesize membrane lipids. In general, overexpression of specific enzymes does not have dramatic effects in overall lipid metabolism because other steps in the pathway become limiting. Therefore, genetic engineering of specific steps probably will have limited impact in overall lipid metabolism. However, genetic modification of a combination of anabolic, catabolic and regulatory steps may lead to profound increases in lipid accumulation.

Further research into the regulatory mechanisms that govern various branches of lipid metabolism is necessary in order to obtain a complete description of the molecular mechanisms that determine fatty acid and lipid accumulation.

All free-living organisms have the machinery to synthesize fatty acids and, conceptually, they could be exploited for biodiesel production. However, the photosynthetic organisms provide the unique opportunity to couple CO_2 sequestration to lipid accumulation and subsequent biodiesel production. In light of the recent genome sequence data and the emergence of synthetic biology we believe that research on the biochemistry and regulation of microbial lipid accumulation is necessary in order to develop competitive technologies for biodiesel production.

Figure Legends.

Figure 1. **Overview of lipid biosynthetic pathways.** Carbon sources are converted to pyruvate which is subsequently converted to acetyl-CoA. Acetyl-CoA enters lipid biosynthesis via the action of acetyl-CoA carboxylase (ACC). Fatty acid biosynthesis proceeds through the action of either type-I (FASI) or dissociated type-II (FASII) systems. The resulting acyl groups are subsequently attached on the glycerol-3-phosphate backbone either via an acyl phosphate intermediate or through the action of specific acyltransferases. PtdOH is distributed between CDP-DAG and DAG. CDP-DAG and DAG are converted mainly to phospholipids via the action of phospholipid synthases. Excess DAG is diverted towards TAG. TAGs can be also derived from phospholipids via the action of PDAT. Abbreviations used: ACC, acetyl-CoA carboxylase; MAT; malonyl-CoA:acyl-carrier-protein transacylase; FASI, type-I fatty acid synthesis system; FASII, type-II fatty acid synthesis system; TES, acyl-acyl carrier protein

thioesterase; PlsX, acyl-phophate synthase; PlsY, acyl-phosphate:glycerol-3-phosphate acyltransferase; GPAT, glycerol-3-phosphate acyltransferase; AGPAT, 1-acylglycerol-3-phosphate acyltransferase; CDS, CDP-diacylglycerol synhtetase; PAP, phosphatidate phosphatase; PLS, phospholipid synthases (referring to variety of enzymes utilizing CDP-DAG and/or DAG for phospholipid synthesis); DGAT, diacylglycerol acyltransferase; PDAT, phospholipid:diacylglycerol acyltransferase; DAG, Diacylglycerol; FFA, Free Fatty Acids; PL, phospholipid; TAG, Triacylglycerol

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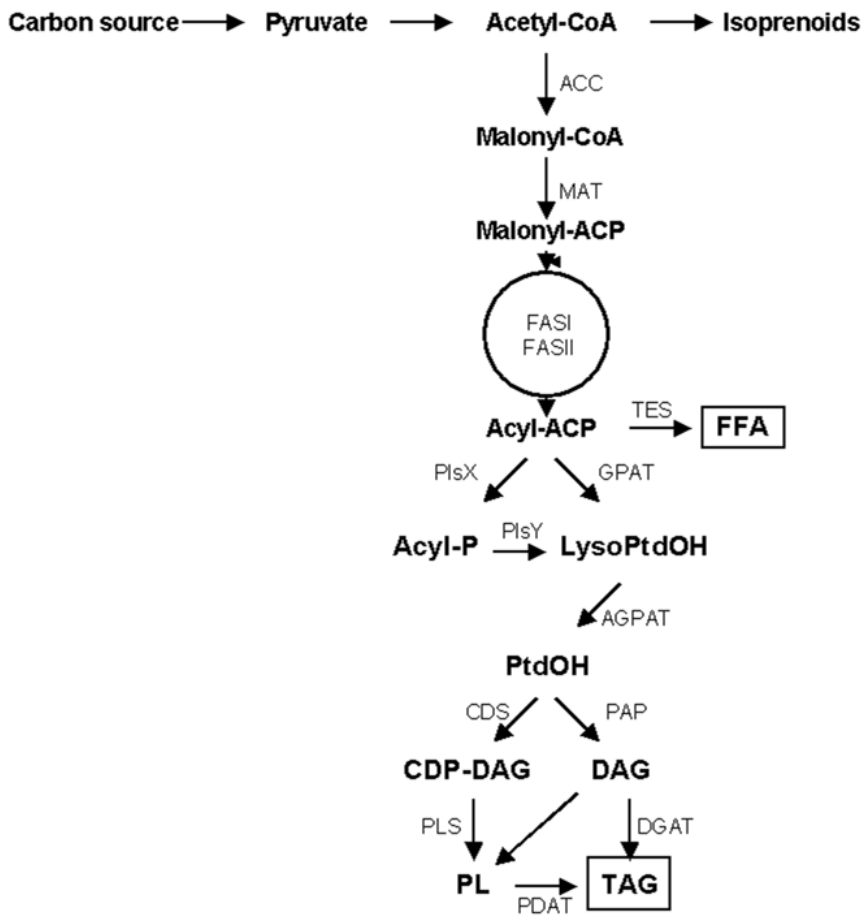
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Isoprenoids
ACC
Malonyl-CoA
MAT
Malonyl-ACP
FASI
FASII
Acyl-ACP
TES
FFA
PlsX
GPAT
Acyl-P
PlsY
LysoPtdOH
AGPAT
PtdOH
CDS
PAP
CDP-DAG
DAG
PLS
DGAT
PL
PDAT
TAG

Figure 1